

# Bacteria in gel probes: comparison of the activity of immobilized sulfate-reducing bacteria with in situ sulfate reduction in a wetland sediment

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## Abstract

A novel method was used to examine the microbial ecology of iron-rich wetland sediments receiving neutral-pH coal mine drainage. Gel probes inserted into the sediments allowed analysis of the distribution and activity of bacterial sulfate reduction (BSR). A mixed population of sulfate-reducing bacteria enriched from anoxic wetland sediments was immobilized in low temperature-gelling agarose held in grooved rods or probes. The probes were inserted vertically into sediments and were allowed to incubate in situ for 48 h. After their retrieval, the gels were sectioned and analyzed for residual BSR activity and were compared to in situ BSR rates and chemical porewater profiles. The depth distribution of residual BSR activity in the immobilized cell gel probes differed significantly from the BSR measured in situ. Approximately 51% of the total integrated residual sulfate reduction activity measured in the gel probes occurred between 0 and 7 cm of the upper 20 cm of sediment. In contrast, ca. 99% of the integrated in situ BSR occurred between 7- and 20-cm depth, and only 1% of the total integrated rate occurred between 0- and 7-cm depth. Lactate-enriched bacteria immobilized in the gel may have been atypical of the majority of sulfate-reducing bacteria in the sediment. Agarose-immobilized sulfate-reducing bacteria might also be able to proliferate in the otherwise inhospitable zone of iron reduction, where sulfate and labile carbon compounds for which they are usually outcompeted can diffuse freely into the gel matrix. Gel probes containing particulate iron monosulfide (FeS) indicated that FeS remained stable in sediments at depths greater than 2 to 3 cm below the sediment-water interface, consistent with the shallow penetration of oxygen into surface sediments. Published by Elsevier Science B.V.

**Keywords:** Gel probes; Immobilized bacteria; Mine drainage; Sulfate reduction; Remediation

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## 1. Introduction

Bacteria are important as the catalysts of a wide variety of geochemical reactions in freshwater and marine sediments. The direct and indirect effects of microorganisms in the oxidation and reduction of

elements such as iron, manganese, and sulfur have been well studied during the past 25 years (Ehrlich, 1990; Lovley, 1995). The bacterial catalysis of these reactions continues to be of interest, especially considering their role in the mobilization and fixation of environmental pollutants and the geochemical behavior of many toxic elements.

Methods of determining in situ bacterial activity in sediments have typically been limited to the removal of sediments from the environment with a

charged by artesian flow from a vertical shaft, traverses a stream channel for 150 m, and enters a 0.8-ha sedimentation pond/natural wetland area prior to merging with Wilson Run (Fig. 1). The stream is a tributary of Sewickley Creek, which in turn feeds into the Youghiogheny River. The study site was located in a wetland area between the outlet of the sedimentation pond and Wilson Run. At this location, the surface sediment consisted of an orange iron oxyhydroxide precipitate. The water depth at the study site was 10 to 15 cm, flow was approximately  $20 \text{ cm s}^{-1}$ , and the water temperature was ca.  $14^\circ\text{C}$ .

Surface water samples were collected over a period of 3 years at the outlet of the study wetland, before it enters Wilson Run. High-density polyethylene sample bottles were cleaned by acid-washing and rinsing with deionized water. Random bottles were selected for blank analysis. Two hundred-milliliter samples for analysis were filtered through  $0.45 \mu\text{m}$  membrane filters and were acidified with 2 ml trace metal-grade concentration HCl to  $\text{pH} < 2$  prior to chemical analysis. Unacidified samples were used for the determination of pH and alkalinity. Measurements of pH were made using an Orion SA270 portable field meter and a combination pH electrode using a two-point calibration. Alkalinity (as  $\text{CaCO}_3$  equivalents) and acidity were determined titrimetrically using US EPA methods 310.1 and 305.1, respectively (U.S. Environmental Protection Agency, 1983).

Sediment cores were taken by hand using 5.5 cm diameter plastic core tubes and were plugged with rubber stoppers. Cores were returned to the lab, sectioned, and subsampled within 2 h of collection. Sediment porewater was obtained by centrifugation under an argon gas atmosphere, filtered through  $0.45 \mu\text{m}$  membrane filters and acidified with trace metal-grade concentration HCl.

## 2.2. Construction of gel probes

Two types of gel probes were constructed, one with immobilized bacteria and one with immobilized FeS particles. In general, aqueous agarose gel solutions were sterilized by autoclaving at  $121^\circ\text{C}$  for 20 min at 15 psi. After the agarose had cooled sufficiently (described below), either live bacterial cells or a suspension of FeS particles was added to the agarose solution. This mixture was then poured into

longitudinal grooves ( $30 \times 0.8 \times 0.6 \text{ cm}$ ) in cylindrical Plexiglas sticks 38-cm long and 2.5 cm in diameter (Fig. 2). Upon solidification of the gel, the probes were ready for deployment.

### 2.2.1. Bacterial gel probes

Sulfate-reducing bacteria for incorporation into gel probes were obtained by adding 2 g of fresh black sediment ( $\text{Eh} < -250 \text{ mV}$ ) obtained at 15-cm depth in a sediment core from the study site to 100 ml of anaerobic API sulfate broth (Difco) and incubating at  $15^\circ\text{C}$  for 7 days. The culture was then centrifuged ( $7000 \times g$  at  $4^\circ\text{C}$ ), washed with an anaerobic mineral salts medium ( $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{NH}_4\text{Cl}$  1 g,  $\text{Na}_2\text{SO}_4$  4.5 g,  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  0.06 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.06 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.004 g; pH 7.0) and centrifuged for three additional times. The final pellet was suspended in 10 ml of anaerobic mineral salts medium. The most probable number (MPN) of bacteria in the suspension was approximated by preparing serial dilutions of the initial suspension in anaerobic API sulfate broth tubes and incubating these at  $15^\circ\text{C}$  for 7 days. The three-tube MPN was determined from statistical tables (Rodina, 1972) based on the formation of a black precipitate as a positive indication of the presence of sulfate-reducing bacteria. Five milliliters of the bacterial suspension were added to 45 ml of 2% (w/v) low temperature-gelling agarose (Type IX-A, Sigma), when the molten agarose had cooled to ca.  $30^\circ\text{C}$ ; final solidification of this agarose occurred at ca.  $17^\circ\text{C}$ . The probes were immediately wrapped with clear polyethylene film and aluminum foil and were placed on ice during transport to the field. Some gels were kept at  $15^\circ\text{C}$  for analysis of initial sulfate reduction activity as described below. Control gels contained 2% agarose in mineral salts medium and contained no added bacteria. All probes were placed in the sediment within 2 h of their construction.

### 2.2.2. FeS gel probes

FeS precipitate was prepared by mixing anoxic aqueous solutions of ferrous iron ( $16.7 \text{ g FeSO}_4 \cdot 7\text{H}_2\text{O}$  per 100 ml) and sulfide ( $14.4 \text{ g Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  per 100 ml) under an argon atmosphere. The black precipitate was gravity filtered and 1 g wet weight (ca. 5 ml slurry) was added to 100 ml 2% agarose/mineral salts solution. The suspension was thoroughly mixed, poured into gel probes, and was al-

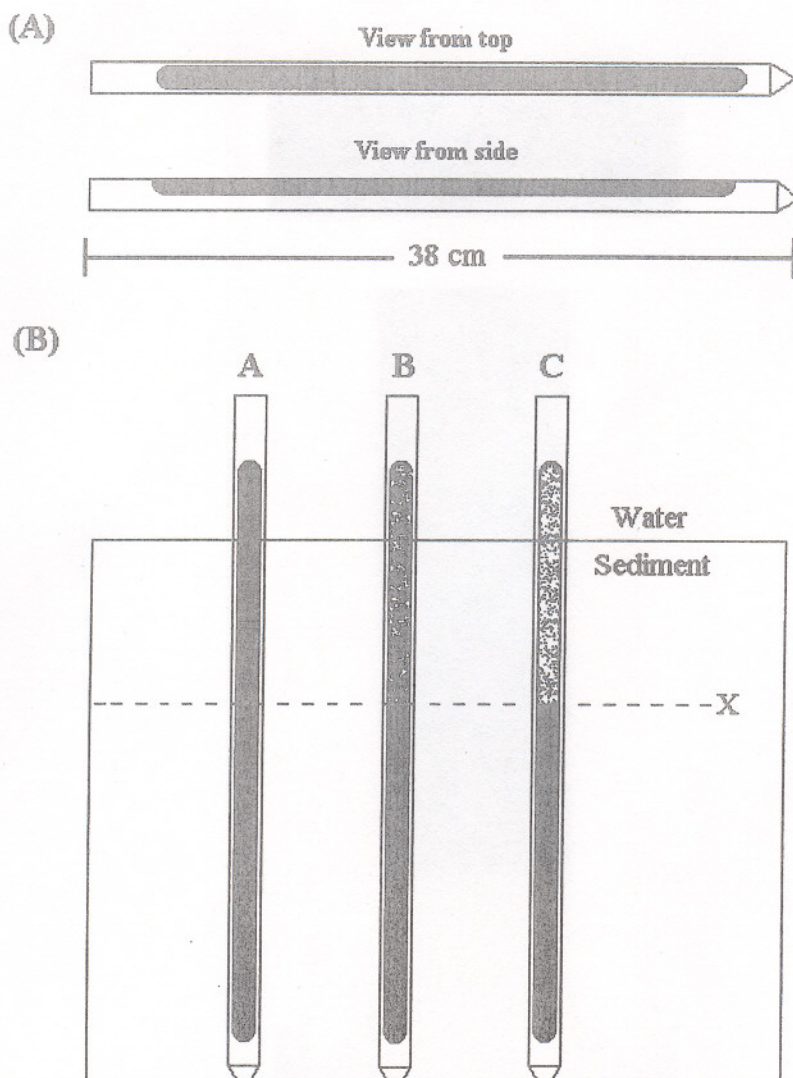


Fig. 2. (A) Schematic of the gel probe containing 2% (w/v) agar with immobilized FeS particles. (B) Diagram showing the method of gel probe placement in the sediment. The appearance of gel probes upon initial insertion (A), after intermediate incubation (B), and after maximum incubation (C) is shown for a reduced particulate substrate such as FeS. X represents the variable and hypothetical sediment depth above which FeS oxidation occurs due to the presence of oxygen in porewater.

lowed to solidify. Probes were transported to the field as described above. Control gels contained 2% agarose/mineral salts solution with no added FeS.

### 2.3. Deployment and incubation of gel probes at field site

Bacterial and FeS gel probes were pushed vertically into the sediment and positioned so that several

centimeters of gel remained above the sediment-water interface, which served as a point of reference (Fig. 2). The distance from the top of the probe to the sediment surface was recorded to detect any rapid sedimentation or erosion during the sampling period. The gels were incubated in situ for 48 h, after which they were removed; any visible color change was immediately observed and measured in the field.

Table 1

Summary of surface water quality at the study site over a 3-year period ( $n = 13$ ). All concentrations except pH are in  $\text{mg l}^{-1}$

	Mean $\pm$ SD	Range
pH	$6.40 \pm 0.17$	6.1–6.65
Alkalinity (as $\text{CaCO}_3$ )	$202 \pm 21$	171–226
$\text{Fe}^{2+}$	$27.1 \pm 1.3$	25–30
Total Fe	$27.5 \pm 1.6$	25–31
$\text{Ca}^{2+}$	$150 \pm 5$	141–160
$\text{Mg}^{2+}$	$49 \pm 1.5$	46–52
$\text{Na}^{2+}$	$82 \pm 3$	76–86
Sulfate	$523 \pm 14$	491–547
$\text{K}^+$	$4.1 \pm 0.6$	3–6

calibration with an equimolar ferricyanide–ferrocyanide solution (precision  $\pm 10$  mV).

### 3. Results

Data summarizing the surface water quality at the study site, collected on random dates over a 3-year period (Table 1) shows that the chemical composition of the water did not vary greatly over this time period, consistent with its origin in flooded underground coal workings and the lack of other significant sources of surface water upstream from the sampling site. The water is characterized by slightly acidic pH, with relatively high iron (principally  $\text{Fe}^{2+}$ ) and sulfate concentrations.

Depth profiles of total dissolved iron in porewater and total iron in the sediment at the study site

(Fig. 3) show distinct peaks and gradients. Total iron (Fig. 3A) exhibited a gradual loss with depth. Porewater iron (Fig. 3B) displayed a subsurface peak in the 5- to 12-cm depth interval, and no porewater iron was found below 12-cm depth. The sediment profile reflects these chemical changes, as the upper 4 to 5 cm were principally orange in color; a deep black color was found at ca. 12-cm depth. The redox potential of the sediments decreased linearly from 100 mV in the surface sediments, to ca.  $-200$  mV at 7-cm depth, to ca.  $-400$  mV at 23-cm depth at the bottom of the core.

Gel probes placed in the sediment (Fig. 4) showed FeS oxidation patterns that reflected the relative flow of surface water in the study area. Oxygen-rich water oxidized immobilized black FeS to an orange color, consistent with the formation of iron oxyhydroxide, from 0- to 2.5-cm subsurface in the gel probes. Apparent oxidation of FeS was slower where surface water current moved slowly, and was greater where the water current was more rapid.

Porewater sulfate concentrations (Fig. 5A) and in situ sulfate reduction rates (Fig. 5B) in the sediment at the study site were compared. Sulfate concentrations were relatively constant in the upper 9 cm of sediment, and then decreased with depth to the bottom of the core at 20 cm. Consequently, in situ sulfate reduction measurements indicated little or no activity in the upper 7 cm, followed by increasing activity down to 14 cm. Below this depth, a drop in sulfate reduction activity was noted, followed by a gradual apparent recovery to peak activity levels by 20-cm depth.

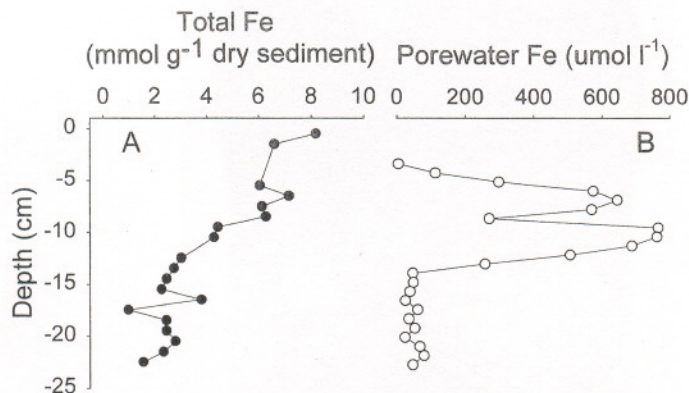


Fig. 3. Depth profiles of (A) total iron and (B) total dissolved iron in sediment porewater at the study site.

the same depth intervals was 51.5% and 48.5%, respectively.

#### 4. Discussion

Sulfate reduction rates measured in the sediments of the study site were comparable to those measured in similar sediments underlying drainage from abandoned pyrite mines (Herlihy and Mills, 1985), constructed wetlands for passive treatment acidic coal mine drainage (McIntire et al., 1990), and a strip mining lake (Blodau et al., 1998). Table 2 compares rates in these different environments and integrated sulfate reduction rates over the top 20 cm of sediment. All of these sites were impacted by mining operations and had elevated concentrations of sulfate, similar to those found in sea water, as opposed to the low concentrations found in freshwaters (Bak and Pfennig, 1991). All of the sites, with the exception of the present study, had very acidic surface waters (Table 2). Acidic surface water has not been found to inhibit sulfate reduction in sediments underlying mine drainage because the bacterial generation of alkalinity in porewaters neutralizes the acidity and maintains a pH conducive to the metabolism of sulfate-reducing bacteria (Herlihy and Mills, 1985; McIntire et al., 1990). The relatively high rates of sulfate reduction in these mine drainage environments have led to the use of constructed wetlands as passive treatment systems to lower dissolved sulfate concentrations, generate alkalinity, and sometimes

precipitate heavy metals (McIntire et al., 1990; Perry and Kleinmann, 1991).

The observed decrease in the porewater sulfate concentration below ca. 9-cm depth correlated well with the observed onset of detectable sulfate reduction at about the same depth (Fig. 5). Blodau et al. (1998) saw a similar subsurface peak between 12- and 18-cm depth in the sediments of the littoral zone of an acidic strip mine lake. The onset of sulfate reduction with depth could be inhibited by the presence and use of alternative electron acceptors by bacteria, such as oxygen and ferric iron. Seasonal deposition of organic matter occurs at the study site, evidenced by layers of partially decomposed leaves at various depths in the sediment cores (data not shown), and these might be expected to influence in situ sulfate reduction rates as well. Bioturbation in the upper sediments might also be expected to result in the upper sediment homogeneity seen in Fig. 5. However, no physical evidence of bioturbation was noted during this study.

Gel-immobilized sulfate-reducing bacteria exhibited residual sulfate reduction activity in the upper sediments of the study site (ca. 0 to 8 cm), as opposed to the in situ sulfate reduction activity, which predominantly occurred below 8-cm depth. This apparent anomaly could be due to one or more reasons. The immobilized bacteria used in this study were enriched from natural sediment on a lactate-based growth medium, so that the incorporated populations would be likely to use lactate as a principal, if not sole, carbon source. Sulfate-reducing bacteria

Table 2  
Comparison of surface water characteristics and maximum and integrated (20 cm) sulfate reduction rates in sediment at four locations impacted by mine drainage

Site	Surface water				Sediment	
	Depth (m)	Temperature (°C)	pH	Sulfate (mM)	Max SRR (nmol cm <sup>-3</sup> day <sup>-1</sup> )	ISR/20 (mmol m <sup>-2</sup> day <sup>-1</sup> )
Contrary Creek, VA <sup>a</sup>	ca. 1	15	3.5	1	550	39
Lake Brandenburg, Germany <sup>b</sup>	3–4	20	< 3	5	130	5
Friendship Hill, PA <sup>c</sup>	0.1–0.2	16	< 3	16	225	NA
Wilson Run, PA <sup>d</sup>	0.1	14	6.4	6	300	23

<sup>a</sup>Herlihy and Mills, 1985. Site C-2; Spring sample.

<sup>b</sup>Blodau et al., 1998. Littoral sample.

<sup>c</sup>McIntire et al., 1990. May sample.

<sup>d</sup>This study. April sample.

remain stable with depth, replacing the tedious task of repeated sediment coring and processing.

## 5. Conclusions

The method described has numerous potential environmental applications, especially in studies where one must delineate the depths over which well-defined geomicrobial populations are active, or to determine the potential toxicity of specific environments at sites to be treated via in situ bioremediation. Despite the contrast in the observed activity between natural sediments and immobilized bacteria incubated in situ, the method demonstrates that it should be possible to use "model" bacteria with known metabolic activity to assess the likely distribution of bacterial activity within the environment or the distribution of biologically toxic compounds.

The application of immobilized microorganism techniques in the environment is in its infancy, and there is much to learn (Cassidy et al., 1996). Where mineral stability alone must be assessed, the gel probe method provides a rapid and inexpensive way to map compound stability over large areas of wetland or other saturated soils, and may be used to assess the potential severity of heavy metal toxicity.

The approach presented in this paper is analogous in many ways to the use of immobilized cells in the construction of microbial biosensors for environmental applications. Biosensors using immobilized whole cells have been developed for the detection of heavy metals (McGrath et al., 1999), cyanide (Lee and Karube, 1996), and general environmental toxicity (Marco and Barceló, 1996). The recent development of biosensors containing recombinant bacterial strains resistant to specific toxic compounds carrying plasmids with firefly luciferase reporter genes (e.g. Tauriainen et al., 1998; McGrath et al., 1999), or other light-emitting mechanisms, suggests that environmental probes incorporating these sensitive detection mechanisms can also be developed.

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